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Apoptosis-like cell death induced by *Salmonella* in *Acanthamoeba rhysodes*

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ABSTRACT

Free-living amoebae act as environmental hosts of several intracellular pathogens. We examined the interaction between *Acanthamoeba rhysodes* and *Salmonella*, a human intracellular pathogen. There was no difference among three different serovars of *Salmonella* in terms of their growth within *A. rhysodes* over time. The number of intracellular bacteria increased at 6 h post-infection, and the viability of *A. rhysodes* was significantly reduced at 24 h post-infection. Amoebic cell death was characterized by TUNEL and Annexin V assay, without DNA ladder identified, indicating an apoptosis-like cell death in *Salmonella*-infected *A. rhysodes*. Global gene expression screening between intracellular and extracellular *Salmonella* by microarray and quantitative PCR showed that genes from *Salmonella* pathogenicity islands and virulence plasmid were up-regulated within *A. rhysodes*. The phase-dependent expression pattern suggests their distinct roles in the pathogenesis. *A. rhysodes* and *Salmonella* provide a model to study transient symbiosis between bacterial pathogens and protozoa in an aquatic ecosystem.

Introduction

Salmonellae are important pathogens in humans and animals. Taxonomically, *Salmonella* organisms are considered as a single species, known as *Salmonella enterica*, which includes more than 2500 different serovars. There are three distinct human disease syndromes caused by *Salmonella*: typhoid fever caused by *S. typhi*, self-limited gastroenteritis by non-typhoid serovars such as *S. typhimurium*, and septicemic diseases without overt diarrhea by *S. choleraesuis*. *Salmonella* pathogenicity islands (SPIs) were considered to play a fundamental role during host cell invasion and intracellular proliferation, two pivotal actions in pathogenesis. It is known that *Salmonella* first remodels the actin cytoskeleton of the host cell and penetrates ileal mucosal lining. Once being intracellular within macrophages, the *Salmonella*-containing vacuole (SCV) was subsequently formed, in which *Salmonella* survives from host defense system and further proliferates.

As a facultatively intracellular pathogen, *Salmonella* can also be isolated from the environment, where they share the habitat with a variety of other bacteria, plants, and protozoa. Free-living amoebae, like *Acanthamoeba* spp., are such a type of microorganism that can be commonly found in natural aquatic systems, soil [1] and even within the intestines of humans [2] and reptiles [3]. *Acanthamoeba* feeds mainly on bacteria and fungi by phagocytosis, and digestion occurs within phagolysosomes. However, *Salmonella* as well as some other human pathogens, such as *Legionella pneumophila* and *Mycobacteria*, is able to survive and replicate in an amoebic intracellular environment and therefore to escape from the predation [4–6]. Moreover, incorporation into amoebic cysts may not only broaden the pathogens' transmission and prevalence but also confer upon the pathogen the enhanced virulence and resistance to adverse environmental conditions [7]. This potential risk urges us to investigate the interaction between *Salmonella* and environmental unicellular host.

Several studies have been made on the interaction between *Salmonella* and human macrophages as well as epithelial cells by microarray approach [8–10]. While the global expression profile of *Salmonella* engulfed by mammalian cells is relatively clear, the knowledge to that of *Salmonella* inside unicellular eukaryotes is nearly blank. It has been reported that *L. pneumophila* uses the same sets of genes for multiplying in human macrophages and amoebic cell [11], so we are keen to see if it is true in *Salmonella*. In this study we established a host–pathogen interaction model using *S. choleraesuis* SC-B67 and *A. rhysodes*, and used a whole

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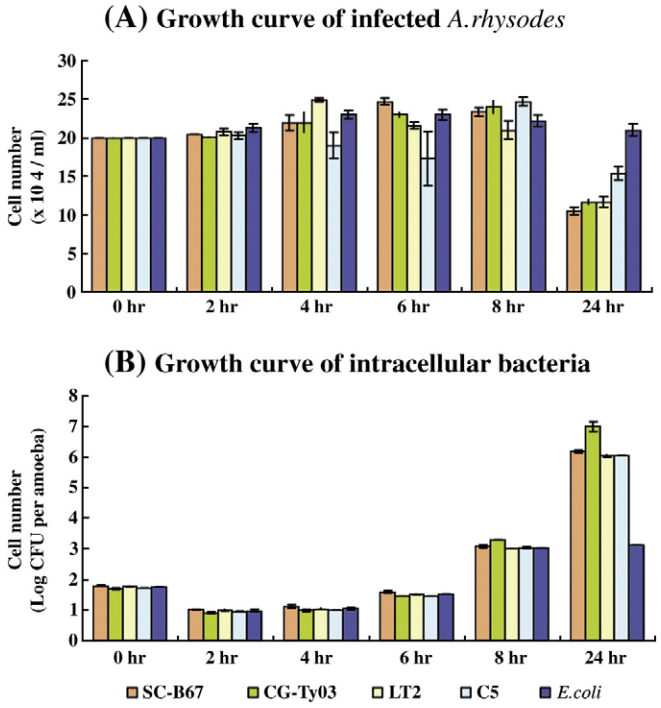

Fig. 1. Growth curve of infected *A. rhysodes* (A) and intracellular bacteria (B). *A. rhysodes* was infected with three serovars of *Salmonella* and *E. coli* individually.

genome oligo array to study the global gene expression profile of intracellular *Salmonella* within *A. rhysodes*.

Results

Growth curve of intracellular Salmonella and viability of A. rhysodes

Our data showed that the viability of *Salmonella*-infected *A. rhysodes* was reduced at 24 h post-infection, but *Escherichia coli*-infected amoebae kept unchanged (Fig. 1). The viability of *A. rhysodes* infected with four different *Salmonella* strains (three serovars) was nearly identical. On the other hand, intracellular *Salmonella* increased at 8 and 24 h post-infection, but the viability of *E. coli* stayed at the same level from 8 to 24 h. Gram stain demonstrated that approximately 12 *Salmonella* bacilli were observed per *A. rhysodes* at 2 and 4 h post-infection and the amoebic cell was filled with bacteria at 6 h post-infection (data not shown).

Detection of amoebic cell death following ingestion of S. choleraesuis SC-B67

Nuclear condensation and DNA laddering are two hallmarks of apoptosis found in multicellular organisms [12,13]. After receiving the external stimuli the nuclear endonuclease is activated and then selectively cleaves DNA, nucleosomal DNA fragments would therefore generate, which resolve on agarose gel electrophoresis as multiple bands of about 180–200 bps. In order to test if the *Salmonella*-induced cell death of *A. rhysodes* fit the criteria of apoptosis, we first examined the presence of DNA fragmentation of *A. rhysodes* after ingestion of *S. choleraesuis* SC-B67. No DNA ladder was found with electrophoresis at 0, 2, 4, 6, 8, and 10 h post-infection. The TUNEL assay was also used to quantify the extent of DNA fragmentation. The result showed that *Salmonella*-infected *A. rhysodes* increased its endonuclease activity from a baseline of 9.87% to 13.70%, 52.00%, and 84.94% at 6, 8, and 10 h post-infection, respectively (Fig. 2).

The exposure of phosphatidylserine (PS) on the outer plasma membrane is another hallmark in apoptotic mammalian cells. Exposed PS can be detected by its affinity to Annexin V, a phospholipids binding protein [14]. The FITC-Annexin V affinity assay showed an increase of exposed PS in infected amoebae from the baseline of 0.32% to 50.6%, 62.8%, and 23.6% at 2, 4, and 6 h post-infection, respectively (Fig. 3). The decrease of PS exposure at 6 h post-infection demonstrated that *Salmonella* began to kill *A. rhysodes* at this time point.

Global gene expression profiles of intracellular Salmonella

We used a whole genome microarray to examine the differential gene expression of *S. choleraesuis* SC-B67 between free-living and intracellular conditions. *A. rhysodes* was infected with *S. choleraesuis* SC-B67 for 2 h. Then all the extracellular bacteria were removed with meropenem treatment for 1 h, and this time point was designated as 0 h post-infection. Three time points, i.e., 2, 8 and 12 h post-infection, were made. At 24 h post-infection the number of *A. rhysodes* was remarkably reduced, so we abandoned the microarray experiment at this time point. To confirm the microarray data, we selected 11 *Salmonella* genes, including those from SPI-1, SPI-2 and flagellar system, for quantitative PCR (Q-PCR) analysis. Of them, 10 showed the same expression pattern in microarray experiments as well as in Q-PCR. The coefficient between microarray data and Q-PCR data is 0.65, suggesting that the microarray data is fairly credible.

Most of the genes involved in differential expression were up-regulated, and the number of down-regulated genes is very small, with the reason unclear. Among the 4433 *Salmonella* genes, 1004

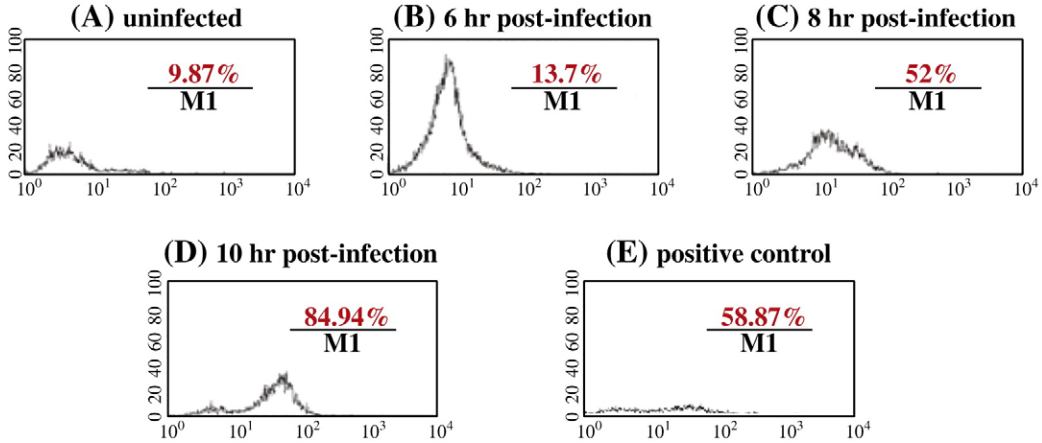

Fig. 2. Analysis of cell death of *S. choleraesuis*-infected *A. rhysodes* by TUNEL assay. (A) Uninfected *A. rhysodes*; (B), (C), and (D) *A. rhysodes* infected with *S. choleraesus* at 6, 8, and 10 h post-infection. (E) Positive control (DNase treatment for 1 h at 37 °C). M1: fluorescence of endonuclease activity; *X*-axis: log fluorescence; *Y*-axis: cell number.

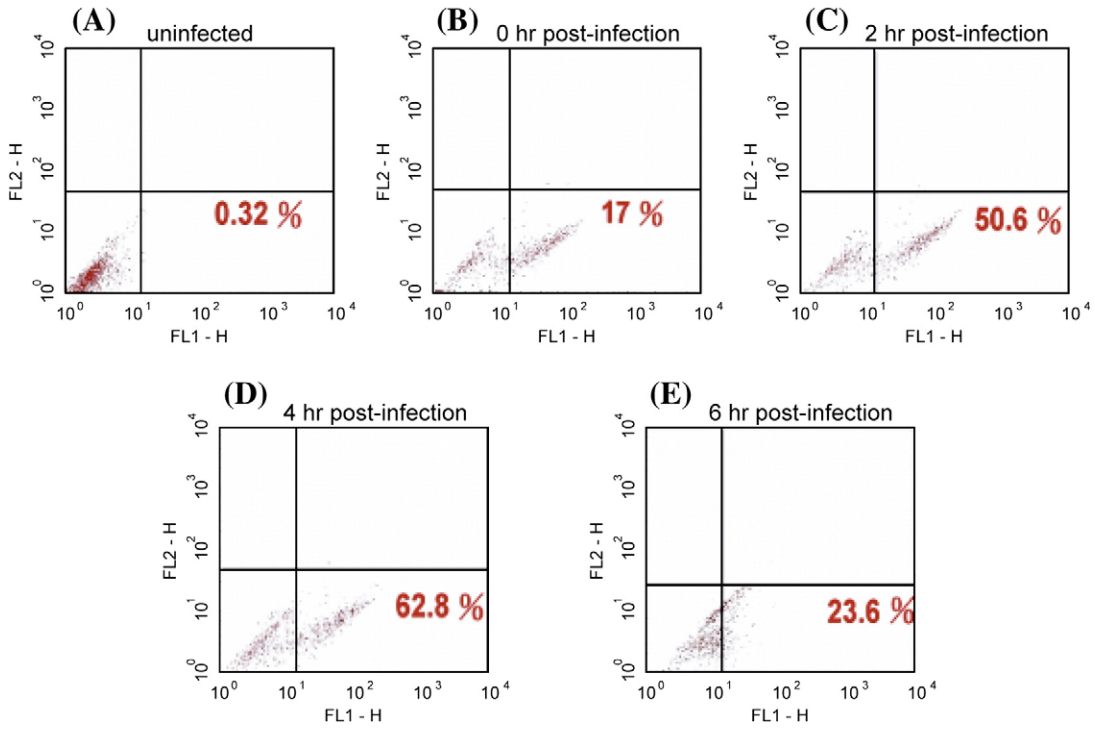

Fig. 3. Annexin V-FITC flow cytometric analysis of exposure of phosphatidylserine in infected *A. rhysodes*. (A) Uninfected control cells; (B–E) Infected *A. rhysodes* at 0, 2, 4, and 6 h post-infection. *S. choleraesuis* infection provokes the exposure of PS in *A. rhysodes*.

genes (22.6%) were up-regulated in intracellular *S. choleraesuis* at one or more time points: 673 genes (15.2%) at only one time point, 107 genes (2.4%) at two time points, and 224 genes (5.0%) at all three time points examined. Besides, the over-expression of *Salmonella* genes was generally higher at 2 h than 8 and 12 h post-infection, and the proportion was 17.1% (760 genes), 7.1% (315 genes) and 13.1% (581 genes), respectively.

Then we analyzed the data from the view of COG (Cluster of Orthologous Groups) functional category [15]. The result is shown in Fig. 4. The number of up-regulated SPI genes was remarkably larger than genes in other COG categories, indicating the important role of these virulence genes. The three time points also show the difference in their COG pattern. Of note is that the category "Translation, ribosomal structure and biogenesis" was exceptionally higher at 12 h than at 2 and 8 h post-infection, revealing that rapid proliferation may occur at this time.

Because a considerable part of differentially expressed genes concentrate within *Salmonella* pathogenicity islands (SPIs), we focus our attention on their expression profile. Within the first sequenced *Salmonella* strain Typhi CT18, a total of ten SPIs have been identified [16]; SPI-11 and SPI-12 were predicted from *S. choleraesuis* SC-B67 genome [17]. SPI-1 and SPI-2 are conserved among the whole *S. enterica* lineage and are also the most extensively studied SPIs. Though both of them encode a Type Three Secretion System (TTSS), their role in pathogenesis is different: SPI-1 is required for invasion into non-phagocytic cells [18] and SPI-2 functions to protect the SCV from the effect of the phagocytic defense enzymes [19]. The known virulence genes in SPI-1 and SPI-2 were up-regulated in all the three time points [see Supplemental Fig. 1]. Other SPIs also contain many genes whose expression levels were increased (Table 1). Interestingly, we observed that these virulence genes have different expression profiles at the three time points. While SPI-1, SPI-4 and SPI-5 genes were expressed at higher levels at 12 h than at 2, 8 h post-infection, SPI-2, SPI-3, SPI-9 and SPI-11 expressed at a higher level at 2 h than at 8, 12 h post-infection. This is perhaps because they play their functions in a phase-dependent fashion.

In addition, we found that flagella genes were all down-regulated during the whole infection process. Flagella genes are responsible for motility of extracellular *Salmonella*. When the bacteria were engulfed within amoebic cell, it is very likely that the flagella have temporarily lost its function. In human macrophage cells flagella genes of *Salmonella* were all down-regulated [8,9]; yet in epithelial cells they were expressed for unknown reasons [10].

Discussion

In the present study, we found that *Salmonella* began to replicate from the 6 h post-infection, and the viability of *A. rhysodes* was reduced by *Salmonella* significantly at 24 h post-infection. No differences were observed between serovars in terms of their intracellular survival. In contrast, *E. coli* str. Top10 did not exhibit equivalent propagation as well as pathogenic potential to *A. rhysodes*. The survival ability of *Salmonella* serovars within amoebic cell described in literature is promiscuous. While someone found evidence that *S. typhimurium* proliferates within *A. rhysodes* [4] and *A. polyphaga* [20], others showed that *S. typhimurium* cannot survive in *A. polyphaga* [21]. Variation between serovars was also observed that *S. dublin* was internalized more efficiently than *S. enteritidis* or *S. typhimurium* [4]. Interaction between *E. coli* and *A. polyphaga* met the same condition [21,22].

We attribute the discrepancy among literature and our result to the differences in the bacteria and amoeba strain used. On one hand, the more pathogenic the bacteria strain is, the stronger its resistance to amoebic phagocytosis. For example, *E. coli* str. Top10 is usually used as a laboratory strain and is less virulent compared to *Salmonella* and pathogenic *E. coli* strains. Both *E. coli* and *Salmonella* belong to Enterobacteriaceae and share over 70% gene content in their genomes, and the major difference between their genome is the pathogenicity islands. All the three *Salmonella* serovars carry SPI-1–SPI-5, which are conserved in all *S. enterica* subspecies I and have been proved contributing to pathogenicity [23]. In contrast, *E. coli* strain Top10 not only lacks these SPIs but also lack pathogenicity islands found in those

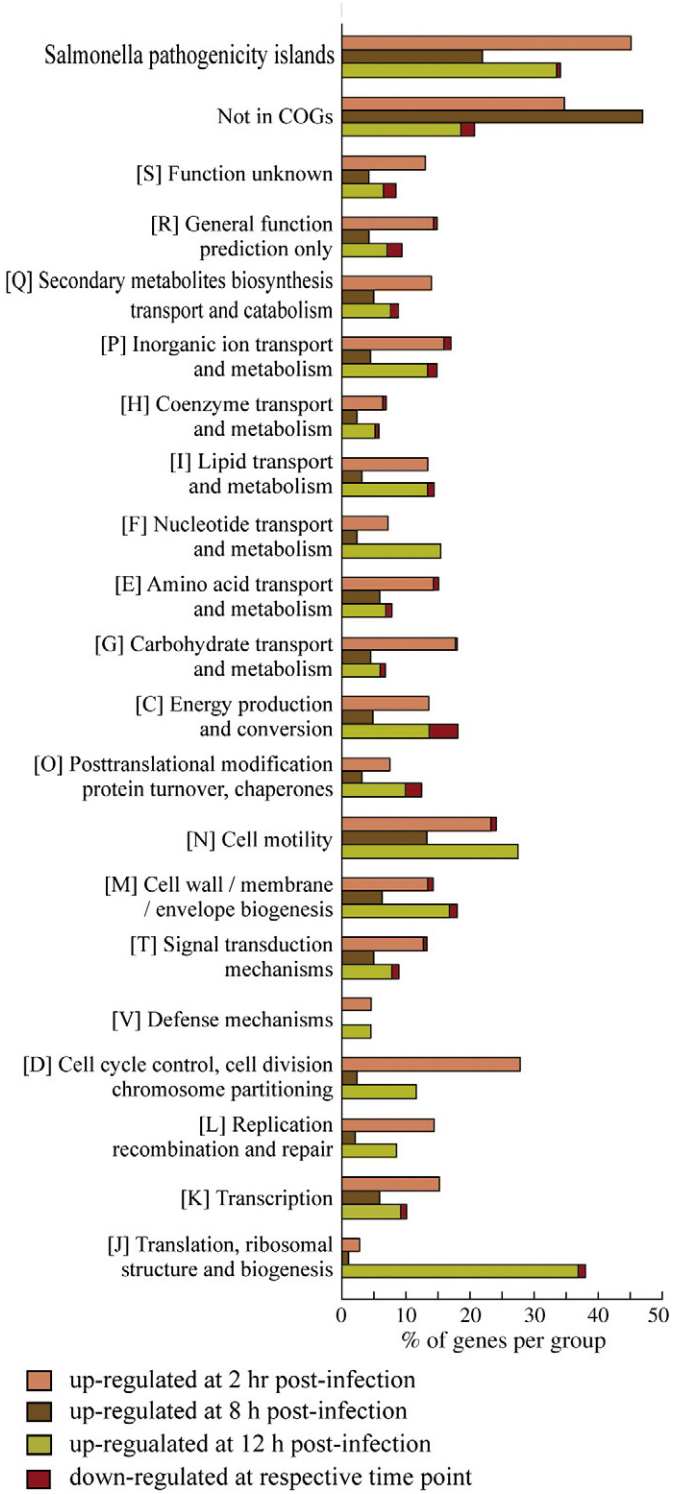

Fig. 4. The percentage of differentially expressed genes in SPIs and COG groups.

pathogenic *E. coli* strains. So we speculate that the strain's virulence property may decide its fate after internalization. On the other hand, the predation ability among *Acanthamoeba* strains is also different. Some *Acanthamoeba* strains would be able to escape killing from some *Salmonella* serovars, whereas some other strains have lost resistance to the bacterial cytotoxicity mediated by all *Salmonella* serovars.

The programmed cell death (PCD) of human macrophages triggered by *Salmonella* has been extensively studied [24]. The *Salmonella*-induced cell death in human microphage can be at least classified into two types. One is often observed rapidly after infection with *Salmonella* (i.e. within 45 min of infection). This type of cell death exhibits features more consistent with necrosis, such as the disruption of plasma membrane and internal organelles and the absence of chromatin condensation. The other type of cell death occurs after prolonged infection and has the typical signs of apoptosis, including chromatin fragmentation, caspase-3 activation, and presence of nucleosomes in the cytoplasm. In this study, we characterized the cell death of infected *A. rhysodes* by TUNEL and Annexin V assays. The amoebic cell death induced by *Salmonella* occurred after 6 h of infection, but oligonucleosomal DNA fragmentation ($\leq$180 bp) and DNA laddering was not observed, which was distinctly different from the above two types of macrophage cell death. However, similar phenomenon has been previously found in other protozoa, i.e., *Blastocystis hominis* and *Dictyostelium discoideum*, which has been denominated "apoptosis-like" cell death [25,26]. In the cell death of the single-celled *D. discoideum*, only perinuclear chromatin condensation and large size DNA fragmentation (>180 bp) could be found, yet without early DNA fragmentation [27]. The whole process of this kind of cell death involves early disruption of mitochondrial transmembrane potential ($\Delta\Psi$m) that precedes the induction of several apoptosis-like features, including exposure of the phosphatidyl residues at the external surface of the plasma membrane, an intense vacuolization, a fragmentation of DNA into large fragments, an autophagy, and the release of apoptotic corpses that are engulfed by neighboring cells. We suggest that the cellular machinery required for PCD may have existed very long ago, even before the advent of multicellularity. While some of the molecules involved in the core PCD mechanism were shared by phylogenetically distant organisms, like unicellular and multicellular eukaryotes, others may be specific to some lineages, which are responsible for DNA laddering and other classical features of PCD found in vertebrates.

Global screening of differentially expressed genes by microarray as well as by Q-PCR between intracellular and extracellular *Salmonella* was analyzed in this study. Most of the differentially expressed genes were up-regulated, and more SPI genes were up-regulated than genes in other COG categories. We compared the expression profile of SPI-1 and SPI-2 in our result with that from experiments on macrophages and epithelial cells [8–10]. Firstly, SPI-1 genes were up-regulated within both human epithelial cells and amoebic cells but down-regulated in macrophage cell. SPI-1 was usually thought helping *Salmonella* with invasion into non-phagocytic cells. So it is easily understood that SPI-1 genes keep silent within macrophage because the bacteria has been inside the cell. The reason why SPI-1 was activated inside epithelial cells and amoebic cells is unknown, perhaps because there are still some SPI-1-inducing cues in the intracellular environment. Secondly, SPI-2 genes were up-regulated within all the macrophage cells, epithelial cells and amoebic cells, suggesting that SPI-2 is indeed required for *Salmonella* survival inside eukaryotic cells. Moreover, SPI-2 genes showed higher expression at 2 h than at 8 and 12 h post-infection. It was reported that the maturation of the SCV is completed by 4 h after phagocytosis, after which bacterial replication begins [28,29]. Thus the initial phase may be the most important moment in which SPI-2 plays its role in the pathogenesis.

Table 1
Proportion of SPI genes of intracellular *S. choleraesuis* within *A. rhysodes* showing increased expression (>2-fold) at different time points.

	2 h	8 h	12 h	Total genes
SPI-1	50%	37%	70%	46
SPI-2	57%	16%	16%	44
SPI-3	30%	7%	15%	27
SPI-4	17%	–[a]	33%	6
SPI-5	33%	33%	50%	6
SPI-9	75%	25%	–	4
SPI-11	53%	35%	35%	17

[a] –, none.

According to the expression profiles of other SPIs, they can be divided into two groups: SPI-3, SPI-9 and SPI-11 genes showed higher expression at 2 h than at 8 and 12 h similar to SPI-2, whereas SPI-4, SPI-5 genes were induced at 12 h rather than at 2, 8 h similar to SPI-1. This phase-dependent pattern found in our study provides potential evidence of their concerted pathogenic role inside *Acanthamoeba*: the intracellular *Salmonella* overcame the survival stress at 6–8 h post-infection by the former SPIs and then caused *A. rhysodes* death at 12 h post-infection presumably by the latter.

Combining the results obtained from the measurement of growth curve, Annexin V assay and microarray data, we reconstructed the sequential order of the whole events after *Salmonella* was ingested by *A. rhysodes*. From 0 h to 6 h post-infection, *Salmonella* keeps quiescent to overcome the survival stress, with the number of *Salmonella* and *A. rhysodes* relatively balanced. From 6 h to 12 h post-infection, *Salmonella* initiated its replication and began to mediate "apoptosis-like" cell death for *A. rhysodes*. Perhaps because *A. rhysodes* still maintained its cell structure at this time, the cell number did not decrease, whereas the number of *Salmonella* increased rapidly. After 12 h post-infection, *A. rhysodes* was killed, and *Salmonella* was released to the environment again.

Materials and methods

Bacterial strains and culture conditions

S. typhimurium strains LT2 and C5, *S. typhi* CG-Ty03, and *S. choleraesuis* SC-B67 were used in this study. *E. coli* K12 strain Top10 was used in this study as a control. All the bacterial strains were grown overnight in LB at 37 °C. Then 1 ml overnight culture was added to 5 ml fresh LB at 37 °C to reach the late logarithmic growth phase prior to the following experiments.

Amoebic cells and culture conditions

The strain of *Acanthamoeba* used in this study was *A. rhysodes* (ATCC50368). It was obtained as an axenic strain and maintained as adherent cells in an axenic culture medium, peptone-yeast extract-glucose (PYG) broth, in 25 cm^3 tissue culture flasks incubated at 25 °C until near-confluence was reached. Amoeba suspension was examined by bright-field microscopy before use, and the number of cells was determined by counting with Burker chamber.

Intracellular bacterial growth in A. rhysodes

A. rhysodes was seeded at the density of 2×10^5 in 5 ml PYG medium at 25 °C for 24 h before bacterial infection. To allow infection, the adhered amoebae were co-incubated with bacteria at a ratio of 500 bacteria per amoebic cell at 25 °C for 2 h. The medium was replaced with fresh PYG medium supplemented with 40 μg/ml meropenem and incubated at 37 °C for 1 h to kill the extracellular bacteria. The cells were then maintained in PYG medium containing 20 μg/ml meropenem. To count the intracellular bacterial number, the amoebic cells were washed twice with PBS and lysed with 0.5% sodium deoxycholate followed by plating the suspension on LB plates. To visualize intracellular bacteria, the *A. rhysodes* suspension was fixed and dried on slides and followed by Gram stain [30]. The viability of *A. rhysodes* was determined at each time point with trypan blue staining, followed by quantification in a Burker chamber.

Isolation and purification of total RNA

At each time point, approximately 3.2×10^6 infected *A. rhysodes* cells were lysed on ice for 30 min with the solution containing 0.1% SDS, 1% acidic phenol, and 19% ethanol, and then the phenol–ethanol mixture was used to stabilize intracellular bacterial RNA [31]. Pellets were collected by centrifugation at 500×*g* for 5 min and the intracellular *Salmonella* RNA was purified according to the manufacturer's instructions (Macherey-Nagel, BmGH and Co. KG, Düren, Germany).

Assessment of nucleosomal DNA

A. rhysodes cells with an initial cell density of 1×10^5 cells/ml were harvested at 0, 2, 4, 6, 8, and 10 h post-infection. DNA was purified following the method described earlier [32]. DNA fragmentation was analyzed by electrophoresis using 2% agarose gel at 100 V for 40 min.

The terminal dUTP nucleotide labeling (TUNEL) assay

TUNEL assay was carried out with the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Cells were seeded at an initial cell density of 1×10^5 cells/ml then infected with *S. choleraesuis* SC-B67. The infected cells and mock control were harvested at 6, 8, and 10 h post-infection. These cells were fixed at room temperature for 1 h in PBS supplemented with 2% paraformaldehyde and then washed once with PBS. Cells were permeabilized for 2 min at 4 °C in solution containing 0.1% Triton X-100 and 0.1% sodium citrate. The labeling and signal conversions were carried out according to the manufacturer's instructions. Finally, samples were analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ) and results were displayed using WinMDI 2.9 software program.

Detection of phosphatidylserine externalization in A. rhysodes

Phosphatidylserine (PS) exposure on the outer plasma membrane of apoptosis cells was detected with Annexin V affinity assay. Cells were infected with the same conditions as described above and harvested at 2, 4, and 6 h post-infection, followed by washing twice with PBS. The labeling and signal conversions were carried out with Annexin V-FITC kit according to the manufacturer's instruction (Abd Serotec, MorphoSys, Munich, Germany). The samples were analyzed using FACScan and results were displayed using WinMDI 2.9 software program.

Microarray procedure

An in-house *Salmonella* oligonucleotide chip covering all coding sequences of *S. choleraesuis* SC-B67 (NC_006905) and *S. typhimurium* LT2 virulence plasmid pSLT (NC_003277) were used in this study [16,17]. Out of 101 genes, only 40 were found in both pSLT and pSCV50 (NC_006855), the 50-kb virulence plasmid of *S. choleraesuis* SC-B67. All the genes had triple spots on the chip and the oligo probes were designed based on the following criteria: they were 70 bp in length and located within the ORF regions; the melting temperature (Tm) was within 80±5 °C; the poly (*N*) tract length was ≦8; and stem length in potential hairpin was ≦9. Approximately 20 μg of total RNA from intracellular *Salmonella* (at 2, 8, and 12 h post-infection) was transcribed to cDNA with Cy dye labeled random hexamers. In order to test the cross-reaction between *Salmonella* and *Acanthamoeba*, their cDNA were labeled with Cy5 and Cy3, respectively, and hybridized to the array. The result showed that less than 1% of probes cross-reacted with each other (data not shown). In the following experiments, cDNA of intracellular *Salmonella* was labeled with Cy3 while cDNA of free-living *Salmonella* was labeled with Cy5. Two biological replicates were made, each with dyeswap replicates. Microarray data were analyzed by using TIGR Microarray Data Analysis System (MIDAS) and Multi-Experiment Viewer (MEV) in TIGR TM4 package (http://www.tm4.org/). Briefly, we used Lowess algorithm to perform normalization. Differentially expressed genes were defined as those which show >2 fold change and were further filtered by the Cross-slide Replicates SAM (Significance Analysis of Microarray) module in MIDAS software.

Reverse transcription and quantitative PCR

Q-PCR was conducted on the same RNA sample to verify the result of microarray experiment. Approximately 5 μg of total RNA isolated from intracellular *Salmonella* was reversely transcribed to first-strand cDNA by random hexamers with Superscript III according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The following genes were selected for this experiment, as they were reported to be associated with *Salmonella*-induced host cell death: *sipB*, *prgI*, *hilA*, *spvR*, *spvB*, *phoP* and *phoQ* [33–35]. Other genes selected were *fliP* for flagella biosynthesis, *ttrB* in SPI-2, *yfcI* in SPI-3, and *phnR*, a probable regulator in an operon controlling 2-aminoethylphosphonate uptake and metabolism [16]. All the primer sequences were listed in Supplemental Table 1. The Q-PCR was performed according to the manufacturer's instructions on iQ5 instrument with SybrGreen Supermix (BioRad Laboratories, Hercules, CA). The final concentration of primers was 0.25 μM in a total volume of 25 μl. The thermal cycling conditions were as follows: 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C and 30 s at annealing temperature (see Supplemental Table 1). The expression of each gene was normalized to *putP*, the expression of which was not affected in different growth conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2009.05.004.

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